'ATENT COOPERATION TREA'

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
T3073(C)/pmk		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 99/ 00481	25/01/1999	26/01/1998
Applicant		
UNILEVER PLC		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [X] furnished subsequently to this Authority in written form.

   [X] furnished subsequently to this Authority in computer readble form.

   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* T3073(C)/pmk

Box No. I TITLE OF INVENTION

METHOD FOR PRODUCING ANTIBODY FRAGMENTS

Box No. II APPLICANT

Name and address: *(Family name followed by given name: for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

UNILEVER PLC

Unilever House, Blackfriars
London, EC4P 4BQ
United Kingdom

☐ This person is also inventor.

Telephone No. (01234) 222893

Facsimile No. (01234) 222633

Teleprinter No.

State *(that is, country)* of nationality: GB

State *(that is, country)* of residence: GB

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☒ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name: for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

UNILEVER NV

Weena 455
3013 AL Rotterdam
Netherlands

This person is:

☒ applicant only

☐ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: NL

State *(that is, country)* of residence: NL

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☒ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name: for a legal entity, full official designation. The address must include postal code and name of country.)*

EVANS, J G V
UNILEVER PLC, Patent Department
Colworth House, Sharnbrook
Bedford, MK44 1LQ
United Kingdom

Telephone No. 01234 222644

Facsimile No. (01234) 222633

Teleprinter No.

☐ Address for correspondence: Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name: for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

HINDUSTAN LEVER LIMITED

Hindustan Lever House, 165/166 Backbay Reclamation
Mumbai 400 020, Maharashtra
India

This person is:
- [X] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: IN

State *(that is, country)* of residence: IN

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [ ] the United States of America only
- [X] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name: for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

FRENKEN, Leo Gerardus Joseph
Unilever Research Vlaardingen
Olivier van Noortlaan 120
3133 AT Vlaardingen
Netherlands

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: NL

State *(that is, country)* of residence: NL

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name: for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

LOGT, Cornelis Paul Erik van der
Unilever Research Colworth
Colworth House
Sharnbrook
Bedford, MK44 1LQ
United Kingdom

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: NL

State *(that is, country)* of residence: GB

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name: for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [ ] the United States of America only
- [ ] the States indicated in the Supplemental Box

- [ ] Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No. V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes: at least one must be marked)*:

Regional Patent

- ☒ AP **ARIPO Patent:** GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SZ Swaziland, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- ☒ EA **Eurasian Patent:** AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- ☒ EP **European Patent:** AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- ☒ OA **OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- ☒ AL Albania
- ☒ AM Armenia
- ☒ AT Austria
- ☒ AU Australia
- ☒ AZ Azerbaijan
- ☒ BA Bosnia and Herzegovina
- ☒ BB Barbados
- ☒ BG Bulgaria
- ☒ BR Brazil
- ☒ BY Belarus
- ☒ CA Canada
- ☒ CH and LI Switzerland and Liechtenstein
- ☒ CN China
- ☒ CU Cuba
- ☒ CZ Czech Republic
- ☒ DE Germany
- ☒ DK Denmark
- ☒ EE Estonia
- ☒ ES Spain
- ☒ FI Finland
- ☒ GB United Kingdom
- ☒ GD Grenada
- ☒ GE Georgia
- ☒ GH Ghana
- ☒ GM Gambia
- ☒ HR Croatia
- ☒ HU Hungary
- ☒ ID Indonesia
- ☒ IL Israel
- ☒ IN India
- ☒ IS Iceland
- ☒ JP Japan
- ☒ KE Kenya
- ☒ KG Kyrgyzstan
- ☒ KP Democratic People's Republic of Korea
- ☒ KR Republic of Korea
- ☒ KZ Kazakhstan
- ☒ LC Saint Lucia
- ☒ LK Sri Lanka
- ☒ LR Liberia
- ☒ LS Lesotho
- ☒ LT Lithuania
- ☒ LU Luxembourg
- ☒ LV Latvia
- ☒ MD Republic of Moldova
- ☒ MG Madagascar
- ☒ MK The former Yugoslav Republic of Macedonia
- ☒ MN Mongolia
- ☒ MW Malawi
- ☒ MX Mexico
- ☒ NO Norway
- ☒ NZ New Zealand
- ☒ PL Poland
- ☒ PT Portugal
- ☒ RO Romania
- ☒ RU Russian Federation
- ☒ SD Sudan
- ☒ SE Sweden
- ☒ SG Singapore
- ☒ SI Slovenia
- ☒ SK Slovakia
- ☒ SL Sierra Leone
- ☒ TJ Tajikistan
- ☒ TM Turkmenistan
- ☒ TR Turkey
- ☒ TT Trinidad and Tobago
- ☒ UA Ukraine
- ☒ UG Uganda
- ☒ US United States of America
- ☒ UZ Uzbekistan
- ☒ VN Viet Nam
- ☒ YU Yugoslavia
- ☒ ZW Zimbabwe

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:

- ☒ Any other states not yet on this form
- ☐ .....
- ☐ .....

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Supplemental Box *If the Supplemental Box is not used, this sheet should not be included in the request.*

*1. If, in any of the Boxes, **the space is insufficient** to furnish all the information: in such case, write "Continuation of Box No. ..." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:*

*(i) **if more than two persons are involved as applicants and/or inventors** and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;*

*(ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication **"the States indicated in the Supplemental Box"** is checked: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;*

*(iii) if, in Box No. II or in any of the sub-boxes of Box No. III, **the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America**: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;*

*(iv) if, in addition to the agent(s) indicated in Box No. IV, there are **further agents**: in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;*

*(v) if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication "**patent of addition**," or "**certificate of addition**," or if, in Box No. V, the name of the United States of America is accompanied by an indication "**continuation**" or "**continuation-in-part**": in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;*

*(vi) if, in Box No. VI, there are **more than three earlier applications whose priority is claimed**: in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI;*

*(vii) if, in Box No. VI, **the earlier application is an ARIPO application**: in such case, write "Continuation of Box No. VI", specify the number of the item corresponding to that earlier application and indicate at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed.*

*2. If, with regard to the **precautionary designation statement** contained in Box No. V, the applicant wishes to exclude any State(s) from the scope of that statement: in such case, write "Designation(s) excluded from precautionary designation statement" and indicate the name or two-letter code of each State so excluded.*

*3. If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning **non-prejudicial disclosures or exceptions to lack of novelty**: in such case, write "Statement concerning non-prejudicial disclosures or exceptions to lack of novelty" and furnish that statement below.*

CONTINUATION OF BOXES NO. II AND NO. III

UNILEVER PLC - Australia, Barbados, Canada, Cyprus (European State), Gambia, Gambia (ARIPO State), Ghana, Ghana (ARIPO State), Grenada, Ireland (European State), Israel, Kenya, Kenya (ARIPO State), Lesotho, Lesotho (ARIPO State), Malawi, Malawi (ARIPO State), Mongolia, New Zealand, Saint Lucia, Sierra Leone, Singapore, Sri Lanka, Sudan, Sudan (ARIPO State), Swaziland (ARIPO State), Trinidad & Tobago, Uganda, Uganda (ARIPO State), United Kingdom, United Kingdom (European State), Zimbabwe, Zimbabwe (ARIPO State)

UNILEVER NV - All designated states except those listed for UNILEVER PLC and HINDUSTAN LEVER LIMITED

HINDUSTAN LEVER LIMITED - India

Box No. VI PRIORITY CLAIM ☐ Further priority claims are indicated in the Supplemental Box.

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) 26 Jan 98 (26.01.98)	98300525.7	United Kingdom	Europe	
item (2)				
item (3)				

☐ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s): _____

* *Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):*

ISA /

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*

Date *(day/month/year)* Number Country *(or regional Office)*

Box No. VIII CHECK LIST; LANGUAGE OF FILING

This international application contains the following **number of sheets**:

request	:	5
description (excluding sequence listing part)	:	20
claims	:	2
abstract	:	1
drawings	:	10
sequence listing part of description	:	
Total number of sheets	:	38

This international application is **accompanied by** the item(s) marked below:

1. ☒ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☒ copy of general power of attorney; reference number, if any: 170
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☐ nucleotide and/or amino acid sequence listing in computer readable form
9. ☐ other *(specify)*:

Figure of the drawings which should accompany the abstract:

Language of filing of the international application: ENGLISH

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

JGVEvans

EVANS, J G V,
European Patent Attorney, G.A. 170

For receiving Office use only

1. Date of actual receipt of the purported international application:	2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:	
4. Date of timely receipt of the required corrections under PCT Article 11(2):	
5. International Searching Authority (if two or more are competent): ISA /	6. ☐ Transmittal of search copy delayed until search fee is paid.

For International Bureau use only

Date of receipt of the record copy by the International Bureau:

This sheet is not part of and does not count as a sheet of the international application.

PCT

FEE CALCULATION SHEET

Annex to the Request

For receiving Office use only

International application No.

Date stamp of the receiving Office

Applicant's or agent's file reference: T3073(C)/pmk

Applicant: UNILEVER PLC, et al

CALCULATION OF PRESCRIBED FEES

1. TRANSMITTAL FEE . . . . . DEM 200.00 [T]

2. SEARCH FEE . . . . . DEM 2200.00 [S]

International search to be carried out by _____

(If two or more International Searching Authorities are competent in relation to the international application, indicate the name of the Authority which is chosen to carry out the international search.)

3. INTERNATIONAL FEE

Basic Fee

The international application contains 38 sheets.

first 30 sheets . . . . . DEM 800.00 [b1]

8 remaining sheets x DEM 19.00 additional amount = DEM 152.00 [b2]

Add amounts entered at b1 and b2 and enter total at B . . . . DEM 952.00 [B]

Designation Fees

The international application contains all designations.

10 number of designation fees payable *(maximum 10)* x DEM 184.00 amount of designation fee = DEM 1840.00 [D]

Add amounts entered at B and D and enter total at I . . . . . DEM 2792.00 [I]

(Applicants from certain States are entitled to a reduction of 75% of the international fee. Where the applicant is (or all applicants are) so entitled, the total to be entered at I is 25% of the sum of the amounts entered at B and D.)

4. FEE FOR PRIORITY DOCUMENT *(if applicable)* . . . . . [P]

5. TOTAL FEES PAYABLE . . . . . DEM 5192.00 TOTAL

Add amounts entered at T, S, I and P, and enter total in the TOTAL box

☐ The designation fees are not paid at this time.

MODE OF PAYMENT

☒ authorization to charge deposit account (see below)	☐ bank draft	☐ coupons
☐ cheque	☐ cash	☐ other *(specify)*:
☐ postal money order	☐ revenue stamps	

DEPOSIT ACCOUNT AUTHORIZATION *(this mode of payment may not be available at all receiving Offices)*

The RO/ EP

☒ is hereby authorized to charge the total fees indicated above to my deposit account.

☒ *(this check-box may be marked only if the conditions for deposit accounts of the receiving Office so permit)* is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.

☐ is hereby authorized to charge the fee for preparation and transmittal of the priority document to the International Bureau of WIPO to my deposit account.

2805.0081 — Deposit Account No.

21/1/99 — Date *(day/month/year)*

Signature: P M KIMBER

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
T3073(C)/pmk		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP99/00481	25/01/1999	26/01/1998
International Patent Classification (IPC) or national classification and IPC C12N15/13		
Applicant UNILEVER PLC et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
20/08/1999	12.05.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Nichogiannopoulou, A Telephone No. +49 89 2399 8054

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP99/00481

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-20 as originally filed

Claims, No.:

1-9 as originally filed

Drawings, sheets:

1/10-10/10 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-9
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-9
Industrial applicability (IA)	Yes:	Claims	1-9
	No:	Claims	

2. Citations and explanations

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Reference is made to the following documents:

   D1: FINNERN, R. et al.: 'Molecular characteristics of anti-self antibody fragments against neutrophil cytoplasmic antigens from human V gene phage display libraries.' CLIN. EXPERIMENTAL IMMUNOLOGY, (1995), 102: 566-574

   D2: ARBABI-GHAHROUDI, M. et al.: 'Selection and identification of single domain antibody fragments from camel heavy chain antibodies.' FEBS LETTERS, (1997), 414: 521-526, cited in the application

   D1 discloses phage display libraries comprising a repertoire of B cell RNA-derived rearranged variable genes from non-immunized humans. Antibody fragments consisting only of a heavy chain variable domain are assembled and can recognise specific antigens.

   D2 discloses the cloning of a repertoire of heavy-chain only antibody variable domains from an immunised dromedary. *Camelidae* immunoglobulins are predominantly homodimers of heavy chains, naturally lacking light chains.

2. **Novelty** (Article 33(2) PCT)
   The present application discloses an expression library comprising a repertoire of single domain binding site "heavy-chain immunoglobulins" derived from a non-immunised donor. Such heavy chains are derived from immunoglobulins naturally devoid of light chains. The subject-matter of claims 1-9 has not been disclosed in the available prior art and is thus considered to satisfy the provisions of Article 33(2) PCT.

3. **Inventive step** (Article 33(3) PCT)
D1 teaches the generation of repertoire libraries from non-immunized subjects, while D2 teaches the generation of heavy-chain-only repertoire libraries from immunized dromedaries. Given the self apparent disadvantages of dromedary immunization, the skilled person would contemplate applying the teaching of D1 (non-immunized subject) to the repertoire library of D2 to arrive at the claimed subject-matter. Claims 1-9 re thus found to lack an inventive step under the terms of Article 33(3) PCT.

4. **Industrial applicability** (Article 33(4) PCT)
The subject-matter of claims 1-9 appear industrially applicable under the terms of Article 33(4) PCT.

Re Item VII
Certain defects in the international application

1. Claim 4 relates to a library wherein "the at least part of the variable domain" is derived from a *camelid* immunoglobulin. The wording of said claim is confusing and requires some clarification.

2. Contrary to the requirements of Rule 5.1(ii) PCT, document D1 is not identified in the description and the relevant background art disclosed therein is not briefly discussed.

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Date of mailing (day/month/year) 25 November 1999 (25.11.99)	
International application No. PCT/EP99/00481	**Applicant's or agent's file reference** T3073(C)/pmk
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Applicant FRENKEN, Leo, Gerardus, Joseph et al	

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INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	R. FINNERN ET AL.: "Molecular characteristics of anti-self antibody fragments against neutrophil cytoplasmic antigens from human V gene phage display libraries." CLINICAL AND EXPERIMENTAL IMMUNOLOGY, vol. 102, no. 3, December 1995 (1995-12), pages 566-574, XP002069901 Oxford, GB the whole document ---	1-9
Y	WO 94 04678 A (CASTERMAN ET AL.) 3 March 1994 (1994-03-03) cited in the application the whole document --- -/--	1-9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	T. VAUGHAN ET AL.: "Isolation of human antibodies with sub-nanomolar affinities directly from a large non-immunized phage display library." IMMUNOTECHNOLOGY, vol. 2, no. 4, November 1996 (1996-11), page 294 XP002069902 Amsterdam, NL left-hand column	1-9
Y	M. ARBABI-GHAHROUDI ET AL.: "Selection and identification of single domain antibody fragments from camel heavy chain antibodies." FEBS LETTERS, vol. 414, no. 3, 15 September 1997 (1997-09-15), pages 521-526, XP002069903 Amsterdam, NL cited in the application discussion abstract	1-9
Y	EP 0 739 981 A (VRIJE UNIVERSITEIT BRUSSEL) 30 October 1996 (1996-10-30) the whole document	1-9
Y	H. GRAM ET AL.: "In vitro selection and affinity maturation of antibodies from a naive combinatorial immunoglobulin library." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE U.S.A., vol. 89, no. 8, 15 April 1992 (1992-04-15), pages 3576-3580, XP000384398 Washington, DC, USA cited in the application abstract figure 2	1-9
A	V. NGUYEN ET AL.: "The specific variable domain of camel heavy chain antibodies is encoded in the germline." JOURNAL OF MOLECULAR BIOLOGY, vol. 275, no. 3, 23 January 1998 (1998-01-23), pages 413-418, XP002069904 London, GB the whole document	1-9

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2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	J. DAVIES ET AL.: "Single antibody domains as small recognition units: design and in vitro antigen selection of camelized, human VH domains with improved protein stability." PROTEIN ENGINEERING, vol. 9, no. 6, June 1996 (1996-06), pages 531-537, XP002069905 Oxford, GB abstract	1-9

INTERNATIONAL SEARCH REPORT

ation on patent family members

International Application No
[redacted]CT/EP 99/00481

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9404678 A	03-03-1994	EP 0584421 A	02-03-1994
		AU 701578 B	04-02-1999
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		AU 9813198 A	25-03-1999
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		EP 0822985 A	11-02-1998

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(54) Title: METHOD FOR PRODUCING ANTIBODY FRAGMENTS

(57) Abstract

An expression library comprising a repertoire of nucleic acid sequences each encoding at least part of a variable domain of a heavy chain derived from an immunoglobulin naturally devoid of light chains and its use in producing antibodies, particularly fragments thereof, is disclosed. The invention provides a method for preparing antibodies, or fragments thereof, having a specificity for a target antigen which avoids the need for the donor previously to have been immunised with the target antigen.

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METHOD FOR PRODUCING ANTIBODY FRAGMENTS

FIELD OF THE INVENTION

The present invention relates to an expression library comprising a repertoire of nucleic acid sequences cloned from a non-immunised source, each nucleic acid sequence encoding at least part of a variable domain of a heavy chain derived from an immunoglobulin naturally devoid of light chains and its use in producing antibodies, or more particularly fragments thereof. In particular, the invention relates to a method for the preparation of antibodies or fragments thereof having binding specificity for a target antigen which avoids the need for the donor previously to have been immunised with the target antigen.

BACKGROUND OF THE INVENTION

Monoclonal antibodies, or binding fragments thereof, have traditionally been prepared using hybridoma technology (Kohler and Milstein, 1975, Nature 256, 495). More recently, the application of recombinant DNA methods to generating and expressing antibodies has found favour. In particular, interest has concentrated on combinatorial library techniques with the aim of utilising more efficiently the antibody repertoire.

The natural immune response *in vivo* generates antigen-specific antibodies *via* an antigen-driven recombination and selection process wherein the initial gene recombination mechanism generates low specificity, low-affinity antibodies. These clones can be mutated further by antigen-driven hypermutation of the variable region genes to provide high specificity, high affinity antibodies.

Approaches to mimicking the first stage randomisation process which have been described in the literature include those based on the construction of 'naive' combinatorial antibody libraries

prepared by isolating panels of immunoglobulin heavy chain variable (VH) domains and recombining these with panels of light variable chains (VL) domains (see, for example, Gram et al, Proc. Natl. Acad. Sa, USA, 89, 3576-3580, 1992). Naive libraries of antibody fragments have been constructed, for example, by cloning the rearranged V-genes from the IgM RNA of B cells of unimmunised donors isolated from peripheral blood lymphocytes, bone marrow or spleen cells (see, for example, Griffiths et al, EMBO Journal, 12(2), 725-734, 1993, Marks et al, J. Mol. Biol., 222, 581-597, 1991). Such libraries can be screened for antibodies against a range of different antigens.

In combinatorial libraries derived from a large number of VH genes and VL genes, the number of possible combinations is such that the likelihood that some of these newly formed combinations will exhibit antigen-specific binding activity is reasonably high provided that the final library size is sufficiently large. Given that the original B-cell pairing between antibody heavy and light chain, selected by the immune system according to their affinity of binding, are likely to be lost in the randomly, recombined repertoires, low affinity pairings would generally be expected. In line with expectations, low affinity antibody fragments (Fabs) with K_as of 10^4-10^5 M^{-1} for a progesterone-bovine serum albumin (BSA) conjugate have been isolated from a small (5×10^6) library constructed from the bone marrow of non-immunised adult mice (Gram et al, see above).

Antibody fragments of higher affinity (K_as of 10^6-10^7 M^{-1} range) were selected from a repertoire of 3×10^7 clones, made from the peripheral blood lymphocytes of two healthy human volunteers (Marks et al, see above) comprising heavy chain repertoires of the IgM (naive) class. These were combined with both Lamda and Kappa light chain sequences, isolated from the same source. Antibodies to more than 25 antigens were isolated from this library, including self-antigens (Griffiths et al, see above) and cell-surface molecules (Marks et al, Bio/Technology, 11, 1145-1149,

1993).

The second stage of the natural immune response, involving affinity maturation of the selected specificities by mutation and selection has been mimicked *in-vitro* using the technique of random point mutation in the V-genes and selecting mutants for improved affinity. Alternatively, the affinity of antibodies may be improved by the process of "chain shuffling", whereby a single heavy or light chain is recombined with a library of partner chains (Marks et al, Bio/Technology, 10 779-782, 1992).

Recently, the construction of a repertoire of 1.4×10^{10} scFv clones, achieved by 'brute force' cloning of rearranged V genes of all classes from 43 non-immunised human donors has been reported (Vaughan et al 1996) and Griffiths et al, see above. Antibodies to seven different targets (including toxic and immunosuppressant molecules) were isolated, with measured affinities all below 10nM.

The main limitation in the construction of combinatorial libraries is their size, which consequently limits their complexity. Evidence from the literature suggests that there is a direct link between library size and diversity and antibody specificity and affinity (see Vaughan et al, Nature Biotechnology, 14, 309-314, 1996), such that the larger (and more diverse) the library, the higher the affinity of the selected antibodies. On this basis, single domain libraries, which omit the process of recombination which is responsible for the generation of variability, would not be expected to be an effective source of high affinity and high specificity antibodies.

EP-B-0368684 (Medical Research Council) discloses the construction of expression libraries comprising a repertoire of nucleic acid sequences each encoding at least part of an immunoglobulin variable domain and the screening of the encoded domains for binding activities. It is stated that repertoires of genes encoding immunoglobulin variable domains are preferably prepared

from lymphocytes of animals immunised with an antigen. The preparation of antigen binding activities from single VH domain, the isolation of which is facilitated by immunisation, is exemplified (see Example 6). Repertoires of amplified heavy chain variable domains obtained from mouse immunised with lysozyme and from human peripheral blood lymphocytes were cloned into expression vectors and probed for lysozyme binding activity. It is reported that 2 positive clones (out of 200) were identified from the amplified mouse spleen DNA and 1 clone from the human cDNA. A library of VH domains from the immunised mouse was screened for lysozyme and keyhole limpet haemocyanin (KLH) binding activities; from 2000 colonies, 21 supernatants were found to have lysozyme binding activity and 2 to have KLH binding activity. An expression library prepared from a mouse immunised with KLH screened in the same manner gave 14 supernatants with KLH binding activity and only 1 with lysozyme binding activity. These results suggest to the Applicants that although antigen binding activities can be seen, these are of very low specificity and affinity (presumably due to the absence of the stabilising effect of the missing light chain such that only half of the designed binding pocket is present, leading to binding with related or homologous targets).

Immunoglobulins capable of exhibiting the functional properties of conventional (four-chain) immunoglobulins but which comprise two heavy polypeptide chains and which furthermore are devoid of light polypeptide chains have been described (see European Patent Application EP-A-0584421, Casterman et al, 1994). Fragments of such immunoglobulins, including fragments corresponding to isolated heavy chain variable domains or to heavy chain variable domain dimers linked by the hinge disulphide are also described. Methods for the preparation of such antibodies or fragments thereof on a large scale comprising transforming a mould or yeast with an expressible DNA sequence encoding the antibody or fragment are described in patent application WO 94/25591 (Unilever).

The immunoglobulins described in EP-A-0584421, which may be isolated from the serum of Camelids, do not rely upon the association of heavy and light chain variable domains for the formation of the antigen-binding site but instead the heavy polypeptide chains alone naturally form the complete antigen binding site. These immunoglobulins, hereinafter referred to as "heavy-chain immunoglobulins" are thus quite distinct from the heavy chains obtained by the degradation of conventional (four-chain) immunoglobulins or by direct cloning. Heavy chains from conventional immunoglobulins contribute part only of the antigen-binding site and require a light chain partner, forming a complete antigen binding site, for optimal antigen binding.

As described in EP-A-0584421, heavy chain immunoglobulin V_H regions isolated from Camelids (forming a complete antigen binding site and thus constituting a single domain binding site) differ from the V_H regions derived from conventional four-chain immunoglobulins in a number of respects, notably in that they have no requirement for special features for facilitating interaction with corresponding light chain domains. Thus, whereas in conventional (four-chain) immunoglobulins the amino acid residue at the positions involved in the V_H/V_L interaction is highly conserved and generally apolar leucine, in Camelid derived V_H domains this is replaced by a charged amino acid, generally arginine. It is thought that the presence of charged amino acids at this position contributes to increasing the solubility of the camelid derived V_H. A further difference which has been noted is that one of the CDRs of the heavy chain immunoglobulins of EP-A-0584421, the CDR_3, may contain an additional cysteine residue associated with a further additional cysteine residue elsewhere in the variable domain. It has been suggested that the establishment of a disulphide bond between the CDR_3 and the remaining regions of the variable domain could be important in binding antigens and may compensate for the absence of light chains.

cDNA libraries composed of nucleotide sequences coding for a

heavy-chain immunoglobulin and methods for their preparation are disclosed in EP-A-0584421. It is stated that these immunoglobulins have undergone extensive maturation *in vivo* and the V region has naturally evolved to function in the absence of the light chain variable domain. It is further suggested that in order to allow for the selection of antibodies having specificity for a target antigen, the animal from which the cells used to prepare the library are obtained should be pre-immunised against the target antigen. No examples of the preparation of antibodies are given in the specification of EP-A-0584421. The need for prior immunisation is also referred to in Arabi Ghahroudi et al (FEBS Letters, 414 (1997), 521-526.

SUMMARY OF THE INVENTION

In a first aspect, the invention provides an expression library comprising a repertoire of nucleic acid sequences cloned from a non-immunised source, each nuceic acid sequence encoding at least part of a variable domain of a heavy chain derived from an immunoglobulin naturally devoid of light chains. Further provided is a method of preparing a cDNA expression library as set forth above comprising providing a repertoire of mRNA from a non-immunised source, treating the obtained RNA with a reverse transcriptase to obtain the corresponding cDNA and cloning the cDNA, with or without prior PCR amplification, into an expression vector. Expression vectors comprising such nucleic acid sequences and host cells transformed with such expression vectors are also provided.

Further provided is the use of a non-immunised source of nucleic acid sequences encoding at least part of a variable domain of a heavy chain derived from an immunoglobulin naturally devoid of light chains to prepare an expression library.

In another aspect, the invention provides a method for the preparation of antibody fragments derived from a non-immunised

source having specificity for a target antigen comprising screening an expression library as set forth above for antigen binding activity and recovering antibody fragments having the desired specificity.

The invention further provides the use of a non-immunised source of nucleic acid sequences encoding at least part of a variable domain of a heavy chain derived from an immunoglobulin naturally devoid of light chains to prepare an antibody, or fragment thereof, having binding specificity for a target antigen.

According to a further aspect, nucleic acid sequences encoding antibody fragments isolated from such a repertoire of variable region genes may be attached to nucleic acid sequences encoding one or more suitable heavy chain constant domains and expressed in a host cell, providing complete heavy chain antibodies.

By means of the invention, antibodies, particularly fragments thereof, having a specificity for a target antigen may conveniently be prepared by a method which does not require the donor previously to have been immunised with the target antigen. The method of the invention provides an advantageous alternative to hybridoma technology, or cloning from B cells and spleen cells where for each antigen, a new library is required.

The present invention may be more fully understood with reference to the following description, when read together with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic representation of the domain structure of the 'classical' four-chain/two domain antibodies (a) and the camelid two chain/single domain antibodies (b).

Figure 2 shows a plasmid map of phage display vector pHEN.5 containing a heavy chain variable domain (HC-V) gene. The DNA and protein sequences of the insertion regions are indicated.

Figures 3A, 3B show a specificity ELISA assay of HC-V-myc samples of clones selected by panning on RR6-BSA (1% gelatin block).

A Specific clones.

B 'sticky' aspecific clones.

RR-6 is an azo dye, available from ICI; BSA is bovine serum albumin; myc is a peptide comprising the sequence Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Asn.

Figure 4 shows inhibition assays of HC-Vs selected by panning on RR6-BSA. Crude HC-V-myc samples were preincubated with increasing concentrations of RR6-BSA, followed by assay of free HC-V-myc on immobilised RR6-BSA.

Figure 5 shows aligned protein sequences of selected anti-RR6 clones. The CDR regions are boxed.

Figure 6 shows a specificity ELISA assay of HC-V-myc samples of clones selected by panning on Dicarboxylic linoleic acid - ovalbumin conjugate (Di-OVA) (1% gelatin block).

Figure 7 shows inhibition of antigen binding activity of the anti-dicarboxylic acid clones D1, D2 and D3 by the presence of free target antigen (Di-OVA) or control conjugate (estrone 3-glucuronide, E3G-OVA).

Figure 8 shows aligned protein sequences of the three selected anti-dicarboxylic clones D1, D2, D3. The CDR regions are boxed.

Figure 9 shows the effect of ammonium thiocyanate (ATC) on binding of HC-Vs to immobilised RR6-BSA. Increasing concentrations of ATC were added to crude HC-V-myc samples bound to immobilised RR6-BSA, followed by detection of remaining bound HC-V using anti-myc monoclonal antibody.

Figure 10 shows the effect of ATC on binding of HC-Vs to immobilised Di-OVA. Increasing concentrations of ATC were added to crude HC-V-myc samples bound to immobilised Di-OVA, followed by detection of remaining bound HC-V using anti-myc monoclonal antibody.

DETAILED DESCRIPTION OF THE INVENTION

The invention is based on the unexpected finding that highly specific antibody fragments against a target antigen may be provided by screening an expression library comprising a repertoire of nucleic acid sequences, each encoding at least part of a variable domain of a heavy chain derived from a non-immunised source of an immunoglobulin naturally devoid of light chains, for antigen binding activity. It would not be predicted that single domain libraries would provide high affinity/high specificity antibodies for the reasons of absence of combinatorial effect discussed above. From the teaching of EP-A-0584421, it would have been expected that in order to produce an antibody specific for a target antigen, either pre-immunisation of the donor with the target antigen or random combination with a VL domain would be necessary.

As used herein, the term "antibody" refers to an immunoglobulin which may be derived from natural sources or synthetically produced, in whole or in part. An "antibody fragment" is a portion of a whole antibody which retains the ability to exhibit antigen binding activity.

A "library" refers to a collection of nucleic acid sequences. The term "repertoire", again meaning a collection, is used to indicate genetic diversity.

The heavy chain variable domains for use according to the invention may be derived from any immunoglobulin naturally devoid of light chains, such that the antigen-binding capability and specificity is located exclusively in the heavy chain variable domain. Preferably, the heavy chain variable domains for use in the invention are derived from immunoglobulins naturally devoid of light chains such as may be obtained from Camelids, as described in EP-A-0584421, discussed above.

Expression libraries according to the invention may be generated using conventional techniques, as described, for example, in EP-B-0368684 and EP-A-0584421. Suitably, a cDNA library comprising a repertoire of nucleic acid sequences each encoding a variable domain of a heavy chain derived from an immunoglobulin naturally devoid of light chains may be generated by cloning cDNA from lymphoid cells, with or without prior PCR amplification, into a suitable expression vector.

Preferably, the nucleic acid sequences used in the method according to the invention are derived from mRNA which may suitably be isolated using known techniques from cells known to produce immunoglobulins naturally devoid of light chains. mRNA obtained in this way may be reacted with a reverse transcriptase to give the corresponding cDNA. Alternatively, the nucleic acid sequences may be derived from genomic DNA, suitably from rearranged B cells.

Suitable sources of heavy chain variable domains derived from immunoglobulins naturally devoid of light chains include lymphoid cells, especially peripheral blood lymphocytes, bone marrow cells, spleen cells derived from camelids.

The nucleic acid sequences encoding the heavy chain variable domains for use according to the invention are cloned into an appropriate expression vector which allows fusion with a surface protein. Suitable vectors which may be used are well known in the art and include any DNA molecule, capable of replication in a host organism, into which the nucleic acid sequence can be inserted. Examples include phage vectors (for example, lambda, T4), more particularly filamentous bacteriophage vectors such as M13. Alternatively, the cloning may be performed into plasmids, such as plasmids coding for bacterial membrane proteins or eukaryotic virus vectors.

The host may be prokaryotic or eukaryotic but is preferably bacterial, particularly *E. coli*.

If the cloned nucleic acid sequences are introduced into an expression vector containing nucleic acid sequences encoding one or more constant domains, heavy chain immunoglobulin chains may be expressed.

Preferably, the cloned nucleic acid sequences may be inserted in an expression vector for expression as a fusion protein.

The expression library according to the invention may be screened for antigen binding activity using conventional techniques well known in the art as described, for example, in Hoogenboom, Tibtech, 1997 (15), 62-70. By way of illustration, bacteriophage displaying a repertoire of nucleic acid sequences according to the invention on the surface of the phage may be screened against different antigens by a 'panning' process (see McCatterty, Nature, 348, (1990), 552-554) whereby the heavy chain variable domains are screened for binding to immobilised antigen. Binding phage are retained, eluted and amplified in bacteria. The panning cycle is repeated until enrichment of phage or antigen is observed and individual phage clones are then assayed for binding to the panning antigen and to uncoated polystyrene by phage ELISA.

Suitable antigens include RR-6 and di-carboxylic linoleic acid.

In accordance with a particular embodiment of the invention, the genes encoding the variable domains of the single domain antibodies of six individual Llamas (which had not been in contact with any of the later used antigens) were isolated and cloned into the phage display vector pHEN which allows the expression of active antibody fragments on the tip of the phage. Eleven libraries (six 'long hinge' and five 'short hinge'), each containing about 10^6 individual members were constructed, together yielding a single 'one-pot' library of approximately 10^7 members with a very high level of complexity.

The library was screened for binding to RR-6 and Di-carboxylic linoleic acid using a panning process. After four and five rounds of panning a significant enrichment was observed for both antigens. After screening individual clones for specific binding activity to its antigen a large number of positive clones were identified via ELISA. Using ELISA technique the clones were shown to be highly active and exhibited strong antigen specific recognition.

The following examples are provided by way of illustration only. Techniques used for the manipulation and analysis of nucleic acid materials were performed as described in Sambrook et al, *Molecular Cloning*, Cold Spring Harbour Press, New York, 2nd Ed. (1989), unless otherwise indicated.

HC-V denotes heavy chain variable domain.

EXAMPLES

EXAMPLE 1. Construction of the naive HC-V library.

1.1 Isolation of gene fragments encoding llama HC-V domains

A blood sample of about 200ml was taken from an non-immunised Llama and an enriched lymphocyte population was obtained via Ficoll (Pharmacia) discontinuous gradient centrifugation. From these cells, total RNA was isolated by acid guanidium thiocyanate extraction (e.g. via the method described by Chomczynnski and Sacchi, (Anal. Biochem, 162, 156-159 (1987). After first strand cDNA synthesis (e.g. with the Amersham first strand cDNA kit), DNA fragments encoding HC-V fragments and part of the long or short hinge region where amplified by PCR using specific primers:

*Pst*I

V_H - 2B 5'-AGGTSMAR**CTGCAG**SAGTCWGG-3'

(see SEQ. ID. NO: 1).

SfiI

PCR.162:5'-

CATGCCATGACTCGCGGCCCAGCCGGCCATGGCCSAGGTSMARCTGCAGSAGTCWGG-3

(see SEQ. ID. NO: 2).

S =C and G, M = A and C, R = A and G , W =A and T,

*Hin*dIII NotI

Lam-07:5'-AACAGTT**AAGCTT**CCGCTTGCGGCCGCGGAGCTGGGGTCTTCGCTGTGGTGCG-3'

(see SEQ. ID. NO: 3).

*Hin*dIII NotI

Lam-08:5'-AACAGTT**AAGCTT**CCGCTTGCGGCCGCTGGTTGTGGTTTTGGTGTCTTGGGTT-3'

(see SEQ. ID. NO: 4).

Upon digestion of the PCR fragments with *Pst*I (coinciding with codon 4 and 5 of the HC-V domain, encoding the amino acids L-Q)

and NotI (located at the 3'-end of the HC-V gene fragments), the DNA fragments with a length between 300 and 400bp (encoding the HC-V domain, but lacking the first three and the last three codons) were purified via gel electrophoresis and isolation from the agarose gel. *Not*I has a recognition-site of 8 nucleotides and it is therefore not likely that this recognition-site is present in many of the created PCR fragments. However, *Pst*I has a recognition-site of only 6 nucleotides. Theoretically this recognition-site could have been present in 10% of the created PCR fragments, and if this sequence is conserved in a certain class of antibody fragments, this group would not be represented in the library cloned as *Pst*I-*Not*I fragments. Therefore, a second series of PCR was performed, in which the primary PCR product was used as a template (10ng/reaction). In this reaction the 5' VH2B primer was replaced by PCR162. This primer introduces a *Sfi*I recognition-site (8 nucleotides) at the 5' end of the amplified fragments for cloning. Thus, a total of 24 different PCR products were obtained, four (short and long hinge, *Pst* I/*Not* I and *Sfi* I/*Not* I) from each Llama. Upon digestion of the PCR fragments with *SfiI* (upstream of the HC-V coding sequence, in the pelB leader sequence) and *NotI,* the DNA fragments with a length between 300 and 400bp (encoding the HC-V domain) were purified via gel electrophoresis and isolation from the agarose gel.

1.2 Construction of HCV Library in pHEN.5

The *Pst* I/*Not* I or *Sfi* I/*Not* I - digested fragments were purified from agarose and inserted into the appropriately digested pHEN.5 vector (Figure 2). Prior to transformation, the ligation reactions were purified by extraction with equal volumes of phenol/chloroform, followed by extraction with chloroform only. The DNA was precipitated by addition of 0.1 volume 3M NaAc pH5.2 and 3 volumes ethanol. The DNA pellets were washed x2 with 1ml 70% ethanol, dried and resuspended in 10 µl sterile milliQ water. Aliquots were transformed into electrocompetent *E.coli* XL1-Blue (Stratagene) by electroporation, using a Bio-Rad Gene Pulser. The protocol used was as recommended by Stratagene. The final

library, consisting of approximately $7.8x10^6$ individual clones, was harvested by scraping the colonies into 2TY + Ampicillin (100ug/ml) + Glucose (2% w/v) culture medium (35-50ml each). Glycerol stocks (30% v/v) and DNA stocks were prepared from these and stored at -80°C.

EXAMPLE 2. Selection of HC-V fragments which exhibit antigen binding affinity.

2.1 Panning of the library

Two 'antigens' were used for screening the naive phage-displayed HCV library;

Di acid-OVA (dicarboxylic linoleic acid-ovalbumin conjugate) and the azo-dye RR6 (available from ICI) conjugated to BSA (reactive red six-bovine serum albumin conjugate).

Phages displaying antibody fragments on their surface were obtained using the following protocol:

Phage rescue:

15mL 2TY/Ampicillin/Glucose was incubated with 100µL of a glycerol stock of the naive library culture. The culture was allowed to grow until log-phase (A_{600}= 0.3-0.5), at which point $4.5x10^9$ pfu M13K07 helper phage were added. After infection for 30 minutes at 37°C (without shaking) the infected cells were spun down (5000 rpm for 10 minutes) and the pellet was resuspended in 200mL 2xTY/Ampicillin/Kan. After incubation with shaking at 37°C overnight, the culture was spun and the paheges present in the supernatant were precipitated by adding 1/5 volume PEG/NaCL (20% Polyethylene glycol 8000, 2.5M NaCL). After incubation on ice-water for 1 hour the phage particles were pelleted by centrifugation at 8000 rpm for 30 minutes. The phage pellet was resuspended in 20mL water and re-precipitated by adding 4mL PEG/NaCl solution. After incubation in ice-water for 15 minutes the phage particles were pelleted by centrifugation at 5000 rpm

for 15 minutes and resuspended in 2mL PBST with 2% Marvel (milk powder; trade name)(plus 2% OVA for the Di acid-OVA tube and 2% BSA for the RR6-BSA tube).

Panning;

The PEG precipitated phages in PBST/2%Marvel (0.5ml) (plus 2% OVA for the Di acid-OVA tube and 2% BSA for the RR6-BSA tube) were added to Nunc-immunotubes (5mL) coated with 1ml Di acid-OVA conjugate (100µg/ml), 1ml RR6-BSA conjugate (100µg/ml) or a control tube. All tubes were blocked with PBST/2% Marvel) (plus 2% OVA for the Di acid-OVA tube and 2% BSA for the RR6-BSA tube) at 37°C for 1 hour before the phages were added. After incubation for 3-4 hours at room temperature, unbound phage were removed by washing the tube 20 times with PBS-T followed by 20 washes with PBS. The bound phages were eluted by adding 1mL elution buffer (0.1M HCL/glycine pH2.2/1mg/mL BSA). The elution mixture was neutralised with 60µL 2M Tris, and the eluted phages were added to 9mL log-phase E.coli XL-1 Blue. Also 4mL log-phase E.coli XL-1 Blue were added to the immunotube. After incubation at 37°C for 30 minutes to allow infection, the 10mL and 4mL infected XL-1 Blue bacteria were pooled and plated onto SOBAG plates (20g bacto-tryptone, 5g bacto-yeast extract, 0.1g Na Cl, 15g Agar; made up to 1 litre with distilled water and autoclaved, allowed to cool and 10mL $MgCl_2$ and 27.8 mL 2M glucose added. Following growth overnight at 37°C the clones obtained from the antigen sensitised tubes were harvested and used as starting material for the next round of panning, or alternatively individual colonies were assayed specific antigen binding activity.

For panning rounds 1 to 3 there was no indication of phage enrichment over background for both antigens (Table 1). However, at pan 4, significant enrichment of phages was observed for both RR6-BSA and Di-acid-OVA.

Table 1. Results of the panning reactions (fold enrichment over background)

Panning Antigen	Pan 1	Pan 2	Pan 3	Pan 4	Pan 5
RR6	none	none	none	100-fold	~200-fold
Di-acid	none	none	none	~100-fold	50-100-fold

EXAMPLE 3. Identification of individual HC-V fragments with antigen binding activity.

Individual bacterial colonies were picked (200 from pans 4 and 5, for both antigens) using sterile toothpicks and added to the wells of 96-well microtitre plates (Sterilin) each containing 100ml of 2TY, 1% (w/v) glucose and ampicillin (100mg/ml). After allowing the cultures to grow overnight at 37°C, 20µl aliquots from each well of these 'masterplates' were added to the wells of fresh microtitre plates each containing 200ml of 2TY, 1% glucose, 100mg/ml ampicillin, 10^9 M13KO7 helper phage. Infection at 37°C for 2.5h was followed by pelleting the cells and resuspending the infected cells in 200ml of 2TY containing ampicillin (100mg/ml) and kanamycin (25mg/ml). Following overnight incubation at 37°C, the phage-containing supernatants (100µl) were added to the wells of Sterilin microtitre plates containing 100µl/well of the appropriate blocking buffer (same buffer used as during panning reactions). Pre-blocking of the phage was carried out in these plates for 30 mins at room temp. After 30 minutes at room temperature, 100µl of phage supernatant was added to the wells of a Greiner HC ELISA plate coated with the corresponding antigen, and to the wells of an uncoated plate. After 2h incubation at 37°C unbound phages were removed, and bound phages were detected with rabbit anti-M13 followed a goat anti-rabbit alkaline phosphatase conjugate. The assays were developed with 100ml/well of *p*-nitrophenyl phosphate (1mg/ml) in 1M diethanolamine, 1mM $MgCl_2$, pH9.6 and the plates read after 5-10 mins at 410nm.

Table 2. Percentage of panned phage clones which specifically recognise and bind immobilised antigen.

Panning Antigens	Pan 4	Pan 5
RR6-BSA	23%	43%
Diacid-OVA	13%	20%

EXAMPLE 4. Characterisation of HC-V fragments with specific RR-6 binding activity.

To test the individual clones identified in the phage ELISA's for their ability to produce active soluble antibody fragments, plasmid DNA from 12 clones that were shown to specifically recognise RR6-BSA was isolated and used to transform the non-suppressor E.coli strain D29AI. Commercially available strains such as TOPIOF (stratagene) and HB2151 (Pharmacia) may alternatively be used. Two transformants of each clone were pre-grown in 10ml 2TY/Ampicillin/Glucose. After 3-4 hours of growth at 37°C (OD_{600}=0.5), the cells were pelleted by centrifugation and resuspended in 5ml 2TY/Ampicillin/IPTG (0.1mM). After 24 hours of incubation at 25°C the cultures were centrifuged, and the supernatants were analysed for the production of antigen binding activity in essential the same way as described in Example 3. In this case, however, the presence of specifically bound HC-V fragments was detected by incubation with monoclonal anti-myc antibodies, followed by incubation with poly-clonal rabbit-anti-mouse conjugate with alkaline phosphatase.

As shown in Figure 3A, six (nR1, nR2, nR5, nR7, nR11 and nR12) out of the twelve chosen RR6-BSA - panned clones were specific for RR6-BSA, and did not bind to any of the other antigens tested. The specificity of these 6 clones was also confirmed in competition assays in which following the protocol outlined above, soluble RR6 or RR6-BSA conjugate was present during the antigen

binding reaction and was shown to reduce the specific binding signal (Figure 4). Another three clones (nR3, nR4 and nR8) were specific for RR6-BSA, but the signals observed were very low. These weak ELISA signals correlated with relatively poor signals in dot-blot experiments, indicating that these clones were poor producers of soluble fragment. This was confirmed by analysis of the supernatants on Western blots (Figure 3B). The remaining 3 clones (nR6, nR9 and nR10) gave significant signals over background on RR6-BSA, BSA and E3G-OVA (Figure 3A). It would appear that these three 'sticky' clones bind to immobilised proteins in general.

The sequence of the isolated anti-RR6 HC-V fragments are listed in Figure 5.

nR1 (SEQ. ID. NO: 5).
nR4 (SEQ. ID. NO: 6).
nR5 (SEQ. ID. NO: 7).
nR8 (SEQ. ID. NO: 8).
nR11 (SEQ. ID. NO: 9).
nR12 (SEQ. ID. NO: 10).

EXAMPLE 5. Characterisation of HC-V fragments with specific Di-Carboxylic Acid binding activity.

To test the individual clones identified in the phage ELISA's for their ability to produce active soluble antibody fragments, plasmid DNA from 9 clones that were shown to specifically recognise Di Acid-OVA was isolated and used to transform the non-suppressor E.coli strain D29AI. Two transformants of each clone were pre-grown in 10ml 2TY/Ampicillin/Glucose. After 3-4 hours of growth at 37°C (OD_{600}=0.5), the cells were pelleted by centrifugation and resuspended in 5ml 2TY/Ampicillin/IPTG (0.1mM). After 24 hours of incubation at 25°C the cultures were centrifuged, and the supernatants were analysed for the production of antigen binding activity in essential the same way as described

in Example 3. In this case, however, 1% gelatin was used as the blocking reagent and the presence of specifically bound HC-V fragments was detected by incubation with monoclonal anti-myc antibodies, followed by incubation with poly-clonal rabbit-anti-mouse conjugate with alkaline phosphatase.

3 of the selected HC-V samples gave high signals against Di acid conjugated to OVA, BSA or PTG (porcine thyro globulin), and background signals against all other immobilised antigens tested (Figure 6). Much lower signals for Di acid-OVA were observed for a further 2 clones (Figure 6). The specificity of the 3 leading clones was further demonstrated using competition assays as described in Example 4, which showed strong inhibition of Di-Acid-OVA binding of these clones when supernatants were preincubated with Di acid-OVA conjugate, whereas the same concentration range of the E3G-OVA conjugate had no inhibitory effect (Figure 7).

The sequence of the isolated anti-Di Acid HC-V fragments are listed in Figure 8.

nD1 (SEQ. ID. NO: 11).
nD2 (SEQ. ID. NO: 12).
nD3 (SEQ. ID. NO: 13).

CLAIMS

1. An expression library comprising a repertoire of nucleic acid sequences cloned from a non-immunised source, each nucleic acid sequence encoding at least part of a variable domain of a heavy chain derived from an immunoglobulin naturally devoid of light chains.

2. A library according to claim 1 wherein the repertoire of nucleic acid sequences is derived from lymphoid cells.

3. A library according to claim 1 or 2 wherein the repertoire of nucleic acid sequences is derived from cDNA clones.

4. A library according to any one of claims 1 to 3 wherein the at least part of the variable domain of a heavy chain is derived from a camelid immunoglobulin.

5. A method of preparing a library according to claim 3 or 4 comprising providing a repertoire of mRNA from a non-immunised source, treating the obtained RNA with a reverse transcriptase to obtain the corresponding cDNA and cloning the cDNA, with or without prior PCR amplification, into an expression vector.

6. Use of a non-immunised source of nucleic acid sequences encoding at least part of a variable domain of a heavy chain derived from an immunoglobulin naturally devoid of light chains to prepare an expression library.

7. A method for preparing antibody fragments derived from a non-immunised source having binding specificity for a target antigen comprising screening an expression library according to any one of claims 1 to 4 for antigen binding activity and recovering antibody fragments having the desired specificity.

8. A method for preparing an antibody derived from a non-immunised source having binding specificity for a target antigen comprising attaching nucleic acid sequences encoding antibody fragments isolated from a library according to claims 1 to 4 to nucleic acid sequences encoding one or more heavy chain constant domains and expressing the product in a host cell.

9. Use of an non-immunised source of nucleic acid sequences encoding at least part of a variable domain of a heavy chain derived from an immunoglobulin naturally devoid of light chains to prepare an antibody fragment having binding specificity for a target antigen.

Fig.1.

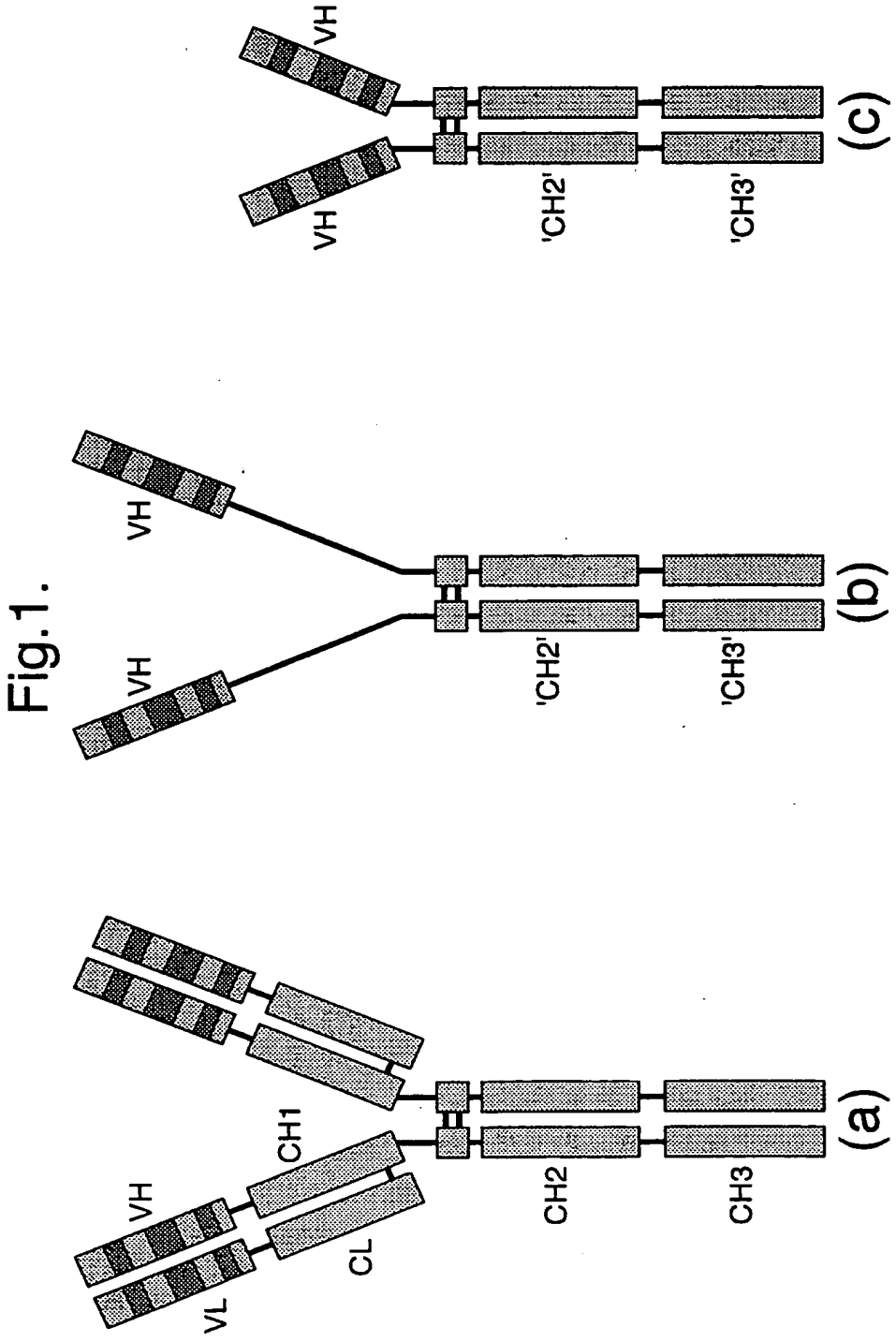
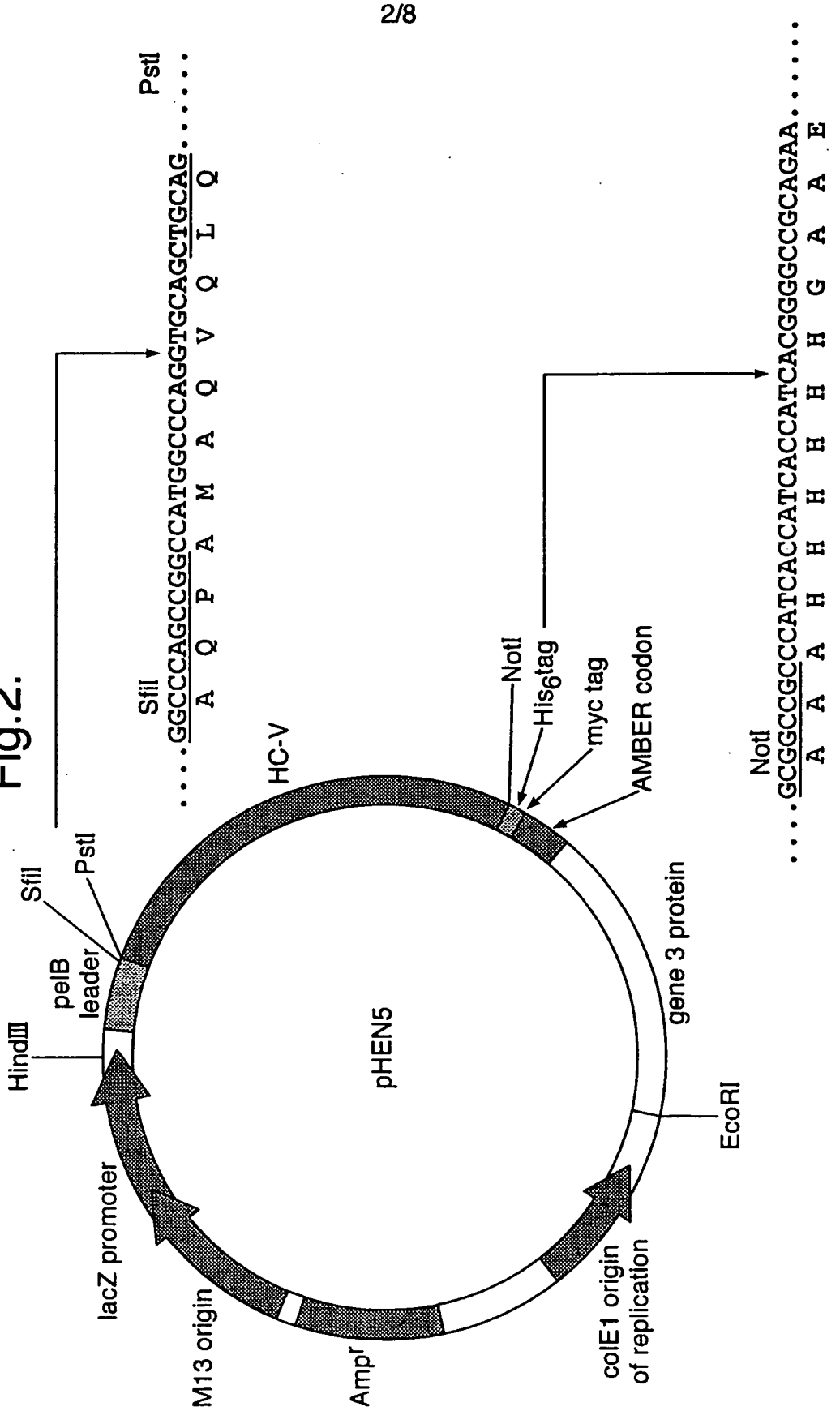


Fig.2.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lacZ promoter
M13 origin
Ampr
colE1 origin of replication
EcoRI
gene 3 protein
AMBER codon
myc tag
His6tag
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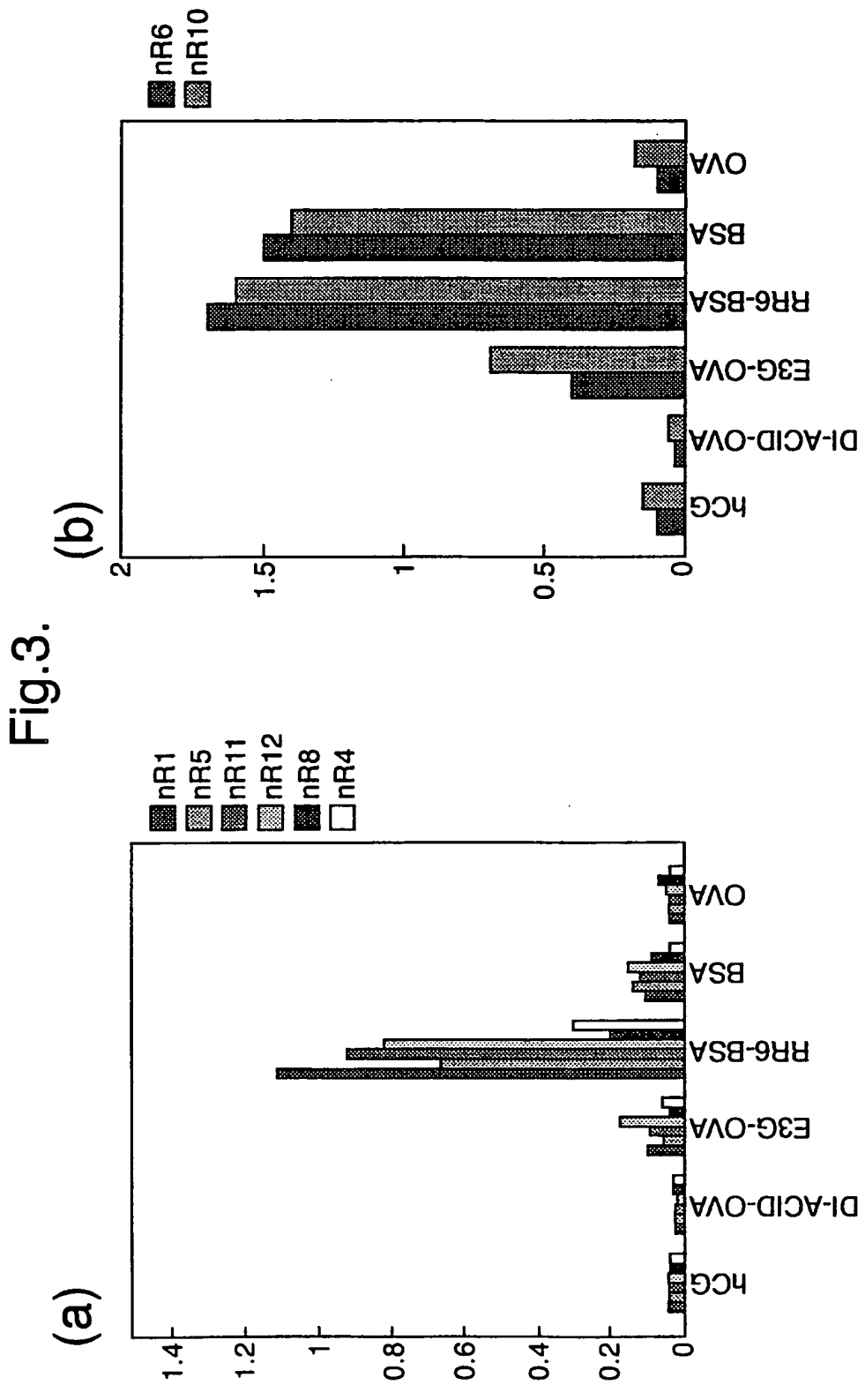
Fig.3.


(b)
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nR10
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BSA
OVA
(a)
nR1
nR5
nR11
nR12
nR8
nR4
1.4
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1
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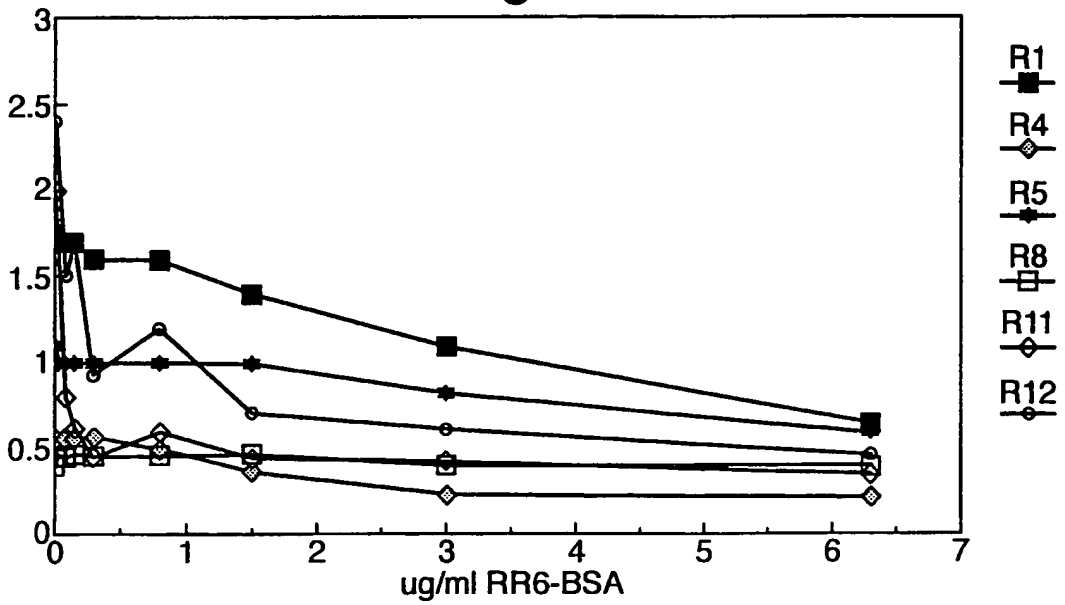
BSA
OVA

Fig.4.



(RR6-BSA on plate)

Fig.7.

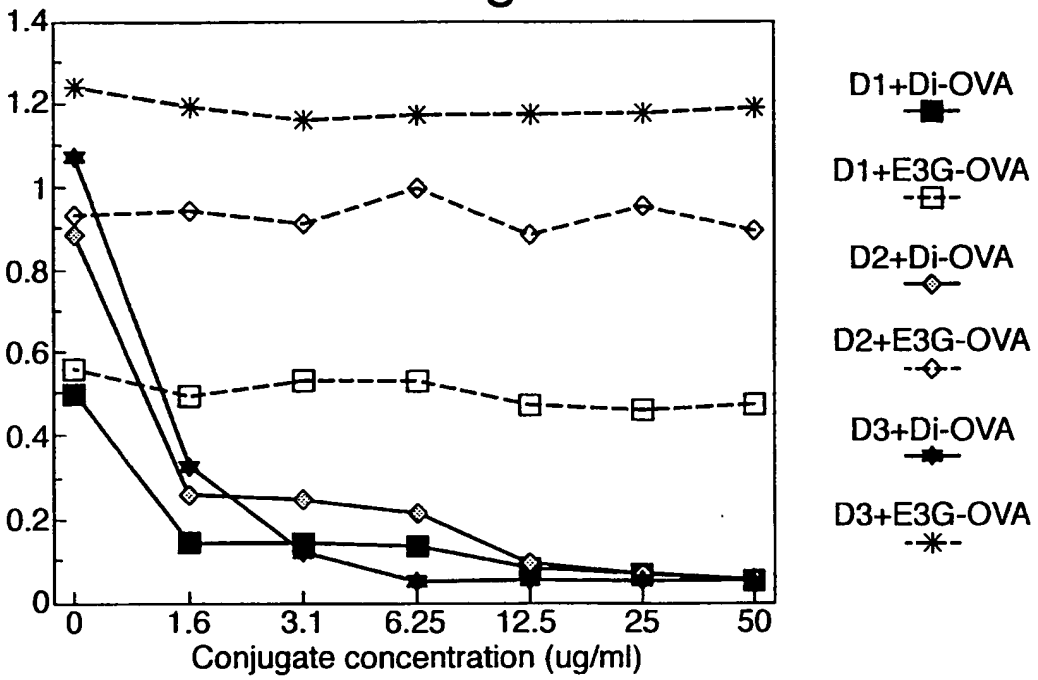


Fig.5.

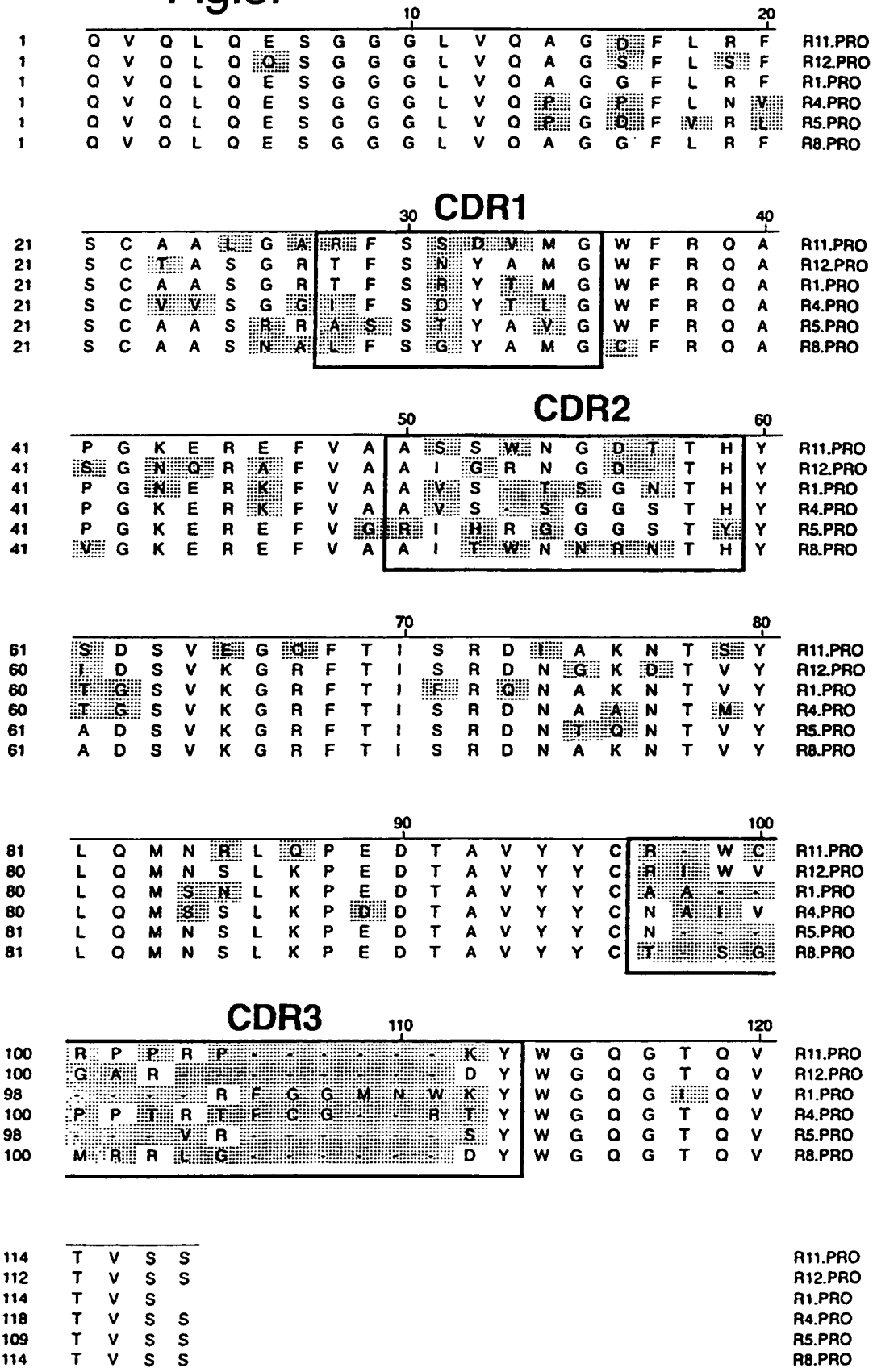
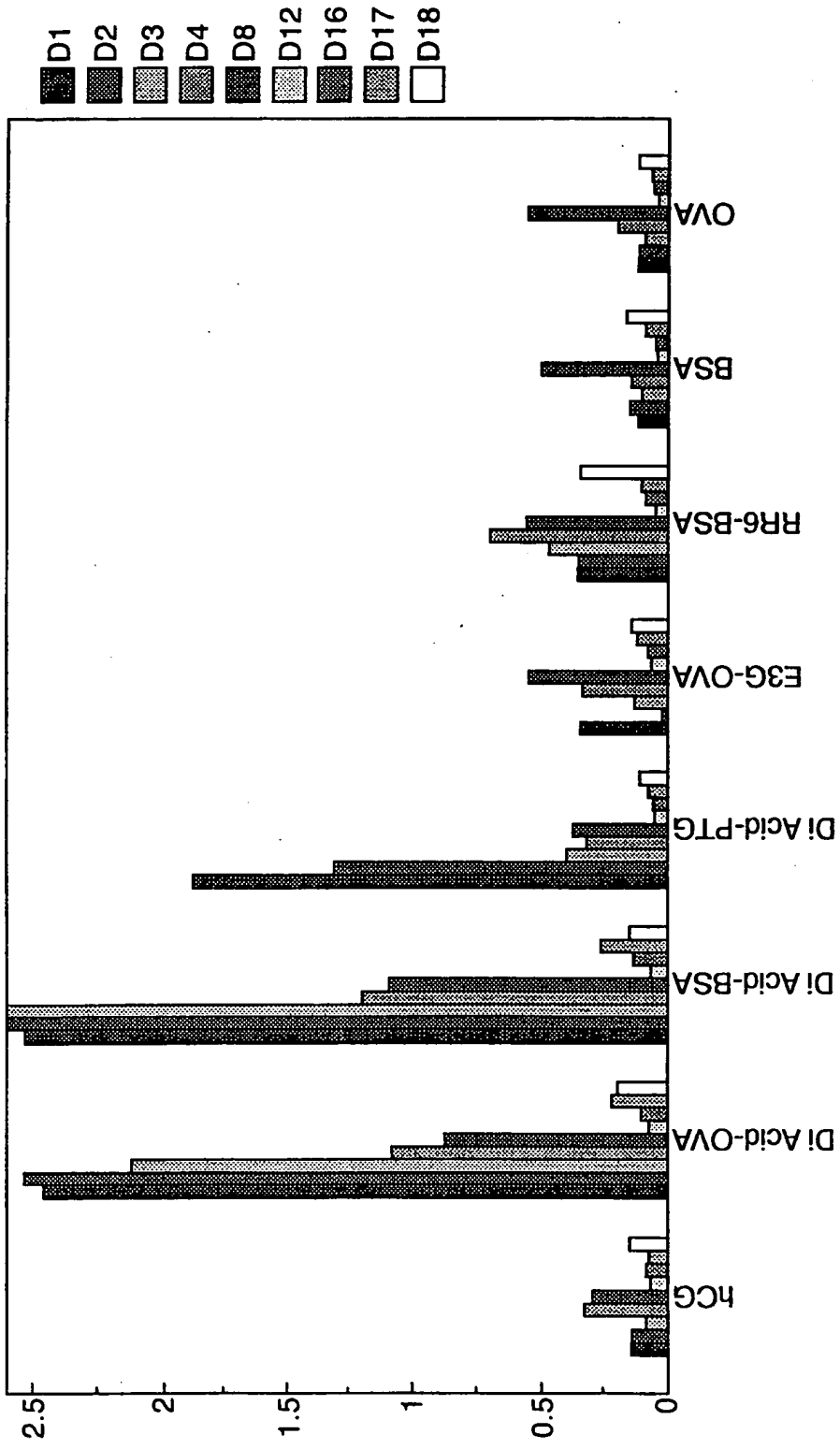


Fig.6.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D2
D3
D4
D8
D12
D16
D17
D18
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BSA
RR6-BSA
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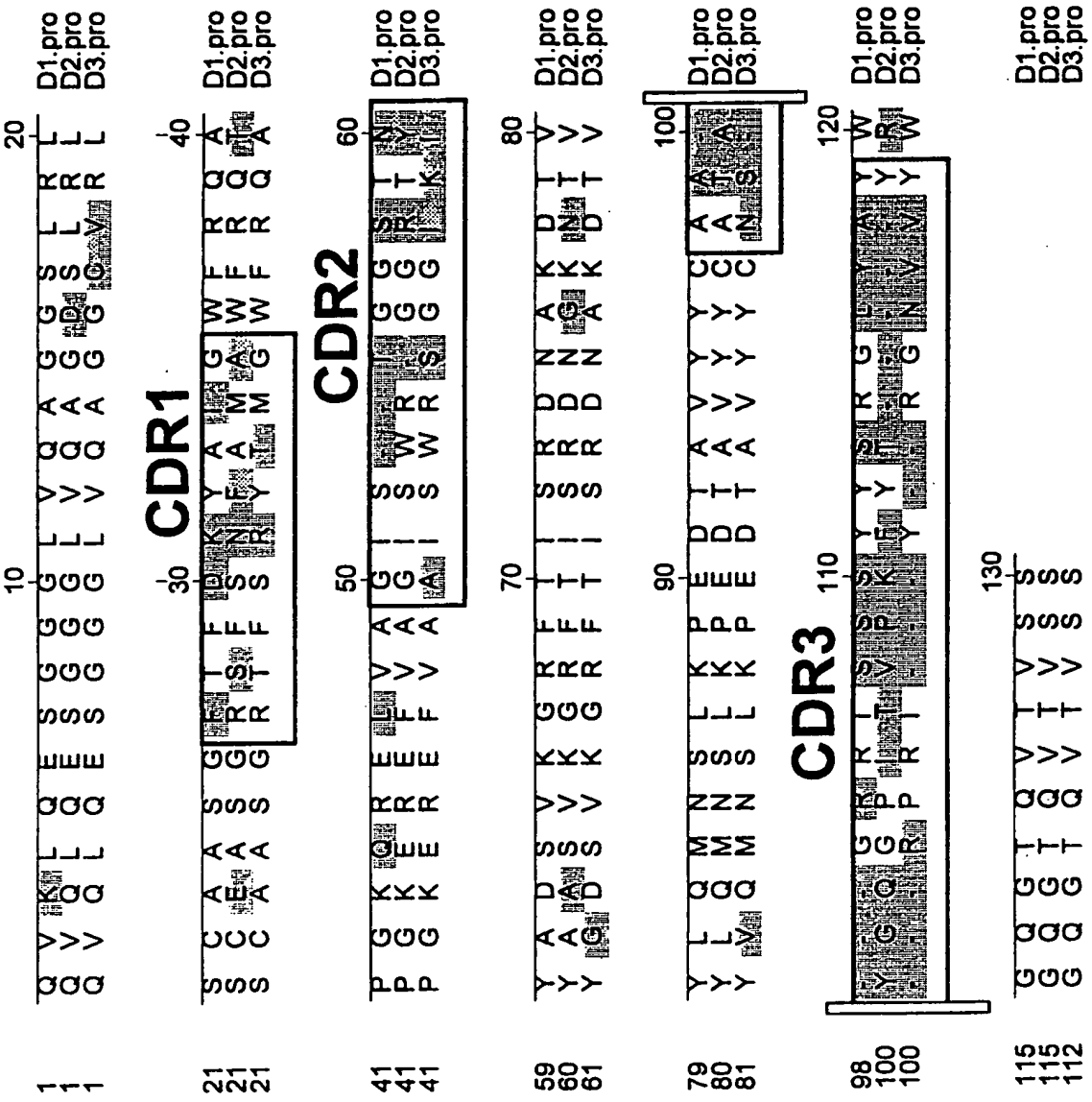
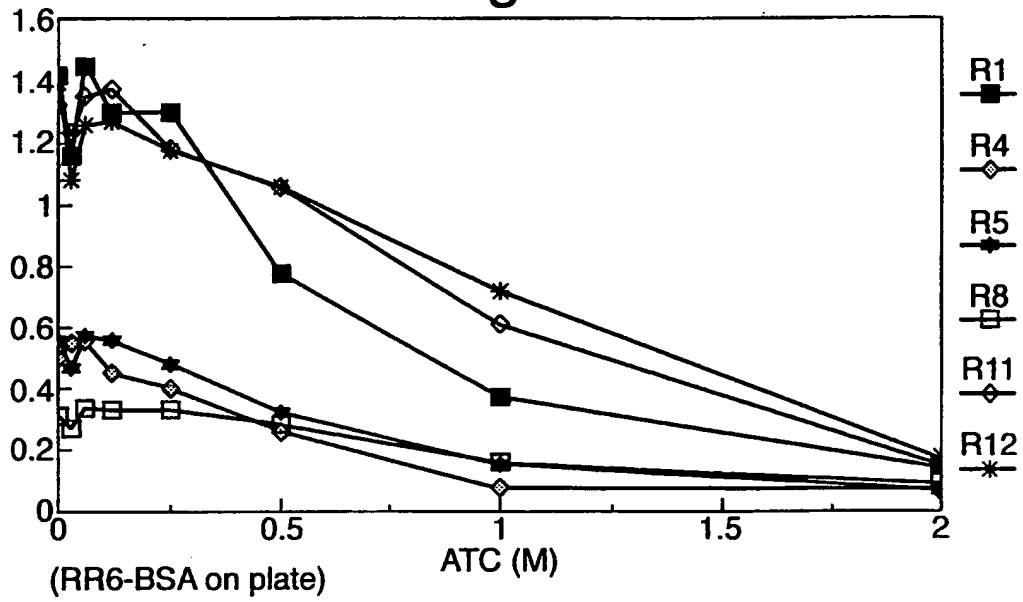


Fig.8.

Fig.9.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0.4
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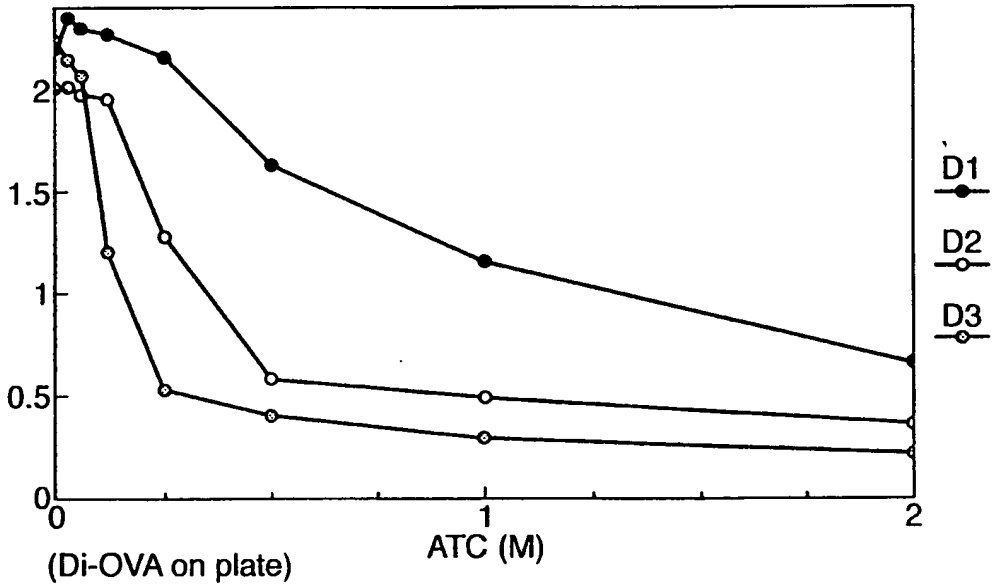
ATC (M)
(RR6-BSA on plate)
R1
R4
R5
R8
R11
R12

Fig.10.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2
ATC (M)
(Di-OVA on plate)
D1
D2
D3